Isozyme Diversity in Wild Red Clover Populations from the Caucasus

Jorge A. Mosjidis,* Stephanie L. Greene, Kimberly A. Klingler, and Alexandr Afonin

ABSTRACT

The amount of genetic variation within species and populations and the distribution of genetic diversity (GD) among populations are important to breeders and biologists. The objective of this study was to assess GD on the basis of isozymes in 15 wild red clover (*Trifolium pratense* L.) populations collected in the Caucasus Mountains, Russia. Isozymes assayed were esterase, β-glucosidase, phosphoglucomutase, peroxidase, diaphorase, phosphoglucoisomerase, and superoxide dismutase. Ten isozyme loci with 26 alleles were detected. Ninety percent of the loci were polymorphic in at least one population. Percent polymorphic loci within populations ranged between 50 and 90%. At the species level, the number of alleles per polymorphic locus was 2.89 and effective number of alleles per locus was 1.84. Within-population averages were 2.75 and 1.70, respectively. Genetic diversity was 0.353 at the species level and the mean value for the populations was 0.323. Some of the populations such as PI 604728 and PI 604735 were unique at the at the isozyme level. The other 13 accessions could be consolidated into one accession. Diversity measurements indicated that this species has a high level of variability that resides mostly within populations. Gene flow expectations based on predictions using topographic maps developed using interpolated digital and climatic surfaces coincided with gene flow estimates obtained using isozymes. This supports the use of GIS tools and remotely sensed data to develop maps that help germplasm collection and post-collection efforts to understand patterns of GD in collected germplasm.

RED CLOVER is one of the most important forage species in the USA and the world. It is a short-lived perennial diploid species that is able to grow in a wide range of soil types, pH levels, and environmental conditions (Smith et al., 1985). It is insect-pollinated and self-incompatible; thus, red clover populations are heterogeneous and consist of heterozygous individuals. Red clover originated in Eurasia and is now widely naturalized throughout the world. However, wild-type populations can be found in remote locations at the center of origin, such as in the Caucasus Mountains, Russia. Its typical natural habitat is meadows, forest margins, and field borders (Gillett and Taylor, 2001).

By understanding the amount of genetic variation within species and populations as well the distribution of genetic diversity among populations, plant breeders and biologists are better able to conserve and utilize plant genetic resources. Using isozyme markers, Hagen and Hamrick (1998) studied nine naturalized red clover populations collected from the U.S. Southeast and Northeast. They measured higher than expected levels of genetic diversity within the populations and low levels of genetic divergence among the red clover populations. The low levels of genetic divergence were attributed to high rates of gene flow evident on a regional geographic scale. The relationship between gene flow and geography was not examined on a smaller geographic scale. Yu et al. (2001) conducted an extensive study on diversity of North American red clover cultivars. Percent polymorphic loci within cultivars ranged from 61.5 to 84.6% with an overall mean of 74.0%. At the species level, the number of alleles per polymorphic locus was 2.55, and effective number of alleles per locus was 1.64. Within-cultivar averages were 2.71 and 1.59, respectively. A high genetic diversity, 0.292 at the species level and 0.285 within cultivars, was measured. Most of the genetic diversity (98.4−99.7%) was distributed within the cultivars.

Greene et al. (1999a) collected wild forage legume germplasm in the western Caucasus Mountains in southern Russia. The collection trip was supported by a Geographic Information System (GIS) database that provided information on topography and climate. Following the collection trip Greene et al. (1999b) used GIS-derived data and local site data to infer the geographic differentiation of 20 red clover populations. These populations had been collected at sites with little apparent disturbance and where no obvious route of dispersal such as roadways or streams were evident. The sites were also not microhabitats, (i.e., the sites were influenced by the broad climatic regime of the area, and so would be described by the GIS data). The objective of the current study was to assess the genetic diversity of 15 of the 20 wild red clover populations by isozyme data. Our intent was to understand better the level and pattern of genetic diversity within and among the red clover populations, and to examine the correspondence of diversity and gene flow with a GIS-derived landscape topography interpolated from data originating from weather stations and remotely sensed sources.

MATERIALS AND METHODS

Eighteen plants, grown from original seed from each of 15 populations of wild red clover collected from diverse habitats in the western Caucasus Mountains, Russia, were grown in pots filled with potting soil in a greenhouse. Isozyme assays were conducted on young leaf tissue from each individual plant. The youngest fully expanded leaf (about 230 mg) was homogenized with 90 μL extraction buffer [sucrose 16.7% (w/v) and sodium ascorbate 8.3%(w/v) in 50 m*M* Tris-HCl, pH 7.4] at −20°C. Crude extracts were centrifuged for 5 min at 8160 × *g*. Supernatant (5.5 μL/well) was loaded onto precast, agarose isoelectric focusing (IEF) gels (Isolab, Akron, OH). Gels with pH gradients 3 to 5 (50%), 3 to 7 (25%), and 3 to 10 (25%) were used for esterase (EST; E.C. 3.1.1.-), β-glucosidase

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(GLU; E.C. 3.2.1.21), phosphoglucomutase (PGM; E.C. 5.4.2.2) and peroxidase (PRX; E.C. 1.11.1.7); gels with pH gradients 3 to 7 (50%) and 4 to 5 (50%) for diaphorase (DIA; E.C. 1.6.99.1) and phosphoglucoisomerase (PGI; E.C. 5.3.1.9); and gels with pH gradients 3 to 10 (75%) and 3 to 7 (25%) for superoxide dismutase (SOD; E.C. 1.15.1.1). Staining procedures were those of Wendel and Weeden (1989) with minor concentration, pH, and ingredient modifications (Yu et al., 2001). The IEF gels were run at constant power and voltage limited to 1500 V. The first run was 60 min at 40 W and the second was 20 min at 60 W.

Population genetic parameters calculated for the species as a whole and on a population basis (indicated by subscripts *s* or *p*, respectively) were percentage of polymorphic loci (*P*), mean number of alleles per polymorphic locus (AP), effective number of alleles per locus (A_e), observed heterozygosity (H_o), and genetic diversity (H_e), which is the expected heterozygosity when populations are allowed to mate randomly (Weir, 1989; Hagen and Hamrick, 1998). Genetic parameters at the population level represent population means, whereas at the species level, parameters represent overall genetic diversity within the species. Mean population parameters were obtained by averaging individual population's *P*, AP, A_e, H_o, and H_e.

Percent polymorphic loci at the population level (P_P) was calculated by dividing the number of loci polymorphic within a population by the total number of loci analyzed. Percent polymorphic loci, at the species level (P_S), was calculated by dividing the number of loci polymorphic in at least one population by the total number of loci analyzed.

Mean number of alleles per polymorphic locus at the population level (AP_p) was determined by summing all the alleles detected at polymorphic loci in a population and dividing by the number of polymorphic loci. The mean number of alleles per polymorphic locus at the species level (AP_S) was determined by summing all the alleles detected at polymorphic loci and dividing by the total number of polymorphic loci. We calculated AP values on the basis of *A* (mean number of alleles per locus).

Effective number of alleles at the population level (A_{eP}) was calculated for each locus by $1/\Sigma f_i^2$ (Hartl and Clark 1997), where f_i is the frequency of the *i*th allele in each population. At the species level, the effective number of alleles was calculated for each locus with the mean frequency of the *i*th allele ($_i$) pooled across all populations. These values were then averaged across loci to obtain A_{eS}.

Genetic diversity was calculated for each locus (including monomorphic and polymorphic loci) by $H_e = 1 - \Sigma f_i^2$. For species values (H_{eS}) f_i is the mean frequency of the *i*th allele ($_i$) pooled across all populations. For population values (H_{eP}) f_i is the frequency of the *i*th allele in each population. Expected heterozygosity was estimated by Nei's (1978) unbiased heterozygosity procedure. Smouse's multilocus test (Smouse et al., 1983) for single populations was used to test each population for Hardy-Weinberg disequilibrium. Population genetics software package POPGENE (Yeh and Boyle, 1997) was used to calculate *P*, A_e, H_o, and H_e and test genetic population parameters.

Population divergence was examined by Nei's genetic identity (Nei 1978) and by Rogers' distance parameters (Wright 1978) for all pairs of populations. Dendrograms based on Rogers' distance were constructed via the unweighed pair group method with arithmetic averages (UPGMA) by the program NTSYS version 2.1 (Rohlf, 2000). This program was employed to calculate the cophenetic value matrix from the tree matrix and to perform the Mantel test (Mantel, 1967) to determine the correspondence between the two matrices. Furthermore, NTSYS was also used to perform a principal components analysis (PCA) on the same data. In addition, the program PHYLIP- Phylogeny Inference Package (Version 3.6a) (Felsenstein, 1993) was used to obtain 1000 data sets by resampling the data using bootstrap. A consensus tree resulting of those 1000 data sets was built with the majority rule, i.e., the tree consisted of all accessions that occurred in more than 50% of the trees was included.

Topographical maps were produced from a digital elevation model that was developed from spot height elevation data at a 500-m horizontal interval. Moisture and temperature zone maps were produced by an unsupervised cluster analysis of seasonal climate surfaces that were interpolated through kriging data obtained from 111 meteorological stations distributed throughout the collection area (see Greene et al. (1999a), for GIS project development). Maps produced from secondarily derived GIS data were used to predict population differentiation based on landscape topography. To confirm the predictions, the populations were grouped by geographic proximity. Nei's G_{ST} (coefficient of gene differentiation) values for each group were calculated according to the formula $G_{ST} = D_{sP}/H_T = (H_T - \overline{H}_{eP})/H_T$, where H_T is total genetic diversity for the group estimated with Nei's genetic diversity statistics (Nei, 1978) and $\overline{H}_{eP}$ is mean diversity within populations. Gene flow (Nm) among populations was estimated from $Nm = 0.25(1 - G_{ST})/G_{ST}$ (Slatkin and Barton, 1989).

The partitioning of genetic diversity was extended to a nested hierarchy of populations by further partitioning H_T (Nei, 1973). Accordingly, H_T was partitioned into diversity among geographic groups (D_R), among populations within groups (D_P), and within populations (H_{wP}) as follows: $H_T = D_R + D_P + {}_{wP}$. Nei's G_{ST} statistics was calculated for each hierarchy level where $G_{sR} = D_R/H_{eS} = (H_{eS} - H_R)/H_{eS}$, and $G_{sP} = D_P/H_{eS} = (H_R - H_{eP})/H_{eS}$, both averaged over all polymorphic loci, represent the proportion of total genetic diversity found among geographic groups and among populations within groups, respectively. The proportion of total genetic diversity found within populations was calculated as $G_{wP} = 1 - G_{ST}$.

RESULTS AND DISCUSSION

A total of 10 isozyme loci with 26 alleles were detected by means of seven enzyme systems in the 15 red clover populations from the Caucasus. Nine of the 10 loci (90%) were polymorphic in at least one population. The diaphorase-2 locus was monomorphic. The percent polymorphic loci (*P*) within populations ranged from 50% in PI 604704 to 90% in PIs 604737 and 604738 with an average of 74% all the populations (Table 1). Percent polymorphic loci at the species level was 90%. Number of alleles per polymorphic locus (*AP*) was 2.89 at the species level and the average within populations was 2.75. They ranged from 2.44 in PI 604738 to 3.01 in PI 604704. The effective number of alleles per locus (A_e) was 1.84 at the species level and the average within populations was 1.70. The A_e values ranged from 1.36 in PI 604735 to 1.89 in PI 604773. Genetic diversity H_e was 0.353 at the species level and the mean value for the populations was 0.323. The H_e value ranged from 0.208 in PI 604735 to 0.381 in PI 604737. The lower H_e value of PI 604735 compared to the other populations suggested that PI 604735 may be a more recently established population since the founder effect can reduce genetic diversity (Nei et al., 1975).

Results reported here indicate that genetic diversity in red clover is high in comparison to most plant species. The average values for isozyme variation of many plant species that are cross-pollinated by animals are *P* =

35.9, AP = 1.54, A_e = 1.17, and H_e = 0.124 (Hamrick and Godt, 1989), whereas the mean values for the wild red clover populations were much higher. The average values for cultivated red clovers were P = 74, AP = 2.71, A_e = 1.59, and H_e = 0.285 (Yu et al., 2001), whereas the wild populations had higher values except for P (Table 1). The reported average values for naturalized red clovers were P = 68, AP = 2.52, A_e = 1.45, and H_e = 0.250 (Hagen and Hamrick 1998), whereas the mean values for the wild red clover populations were higher.

Hardy-Weinberg expectations for genotypic frequencies were not observed in 27 of the 135 (15 populations times nine polymorphic loci) chi-square tests. Considering that 6.75 significant deviations at the $P = 0.05$ significance level would be expected by chance alone, the 15 populations as a whole were not in Hardy-Weinberg equilibrium. However, Smouse's multilocus test for single populations detected that nine of 15 populations did not deviate ($P < 0.05$) from random union of gametes; thus, these populations should be in Hardy-Weinberg equilibrium. Those populations are PI 604704, PI 604710, PI 604712, PI 604713, PI 604728, PI 604735, PI 604737, PI 604739, and PI 604771. Allele frequencies were significantly different among populations for five of the nine polymorphic loci ($P < 0.001$). The exceptions were *Dia-1, Glu*, *Pgm*, and *Prx-2*.

Genetic identity values between pairs of populations ranged between 1.000 and 0.835. This range is wider than the one reported for cultivated red clover (Yu et al., 2001). The highest value was 1.000 for populations PI 604738 and PI 604741 and the lowest values were for PI 604734 and PI 604735 (0.835) and for PI 604728 and PI 604713 (0.843) and PI 604728 and PI 604734 (0.849). The average genetic identity was 0.955.

Table 1. Summary of isozyme variation within 15 Caucasus populations of *T. pratense* and at the species level. Parameters calculated on the basis of 10 loci were percent polymorphic loci (*P*), mean number of alleles per polymorphic locus (AP), effective number of alleles per locus (A_e), observed heterozygosity (H_o), and expected heterozygosity (H_e).

Entries	*P*	AP	A_e	H_o	H_e
PI 604697	80	2.63	1.79	0.402	0.372
PI 604704	50	3.01	1.45	0.321	0.232
PI 604710	80	2.63	1.69	0.309	0.329
PI 604712	80	2.63	1.78	0.359	0.337
PI 604713	80	2.75	1.76	0.313	0.337
PI 604728	60	2.84	1.53	0.319	0.297
PI 604734	70	3.00	1.69	0.308	0.313
PI 604735	60	2.78	1.36	0.265	0.208
PI 604737	90	2.56	1.87	0.380	0.381
PI 604738	90	2.44	1.84	0.404	0.364
PI 604739	80	2.75	1.66	0.332	0.310
PI 604740	70	2.86	1.78	0.386	0.349
PI 604741	80	2.75	1.79	0.399	0.335
PI 604771	70	2.86	1.65	0.359	0.296
PI 604773	70	2.86	1.89	0.401	0.380
mean	74	2.75	1.70	0.350	0.323
SD	11	0.16	0.15	0.04	0.05
Species	90	2.89	1.84	0.354	0.353

Cophenetic correlation, i.e., the correlation between the cophenetic matrix and the matrix based on Rogers' distance, was r = 0.90 indicating a very good fit to the dendrogram derived from the cluster analysis. The dendrogram of genetic distance indicated that PI 604738 and PI 604741 that were alike and that PI 604728 and PI 604735 were substantially different from the other 13. Those two populations, PI 604728 and PI 604735, grouped separately from the rest of the populations. In fact, the dendrogram suggested three clusters, one containing 13 populations and two clusters each containing one population, PI 604728 and PI 604735, respectively (Fig. 1). These two accessions were collected at high elevation

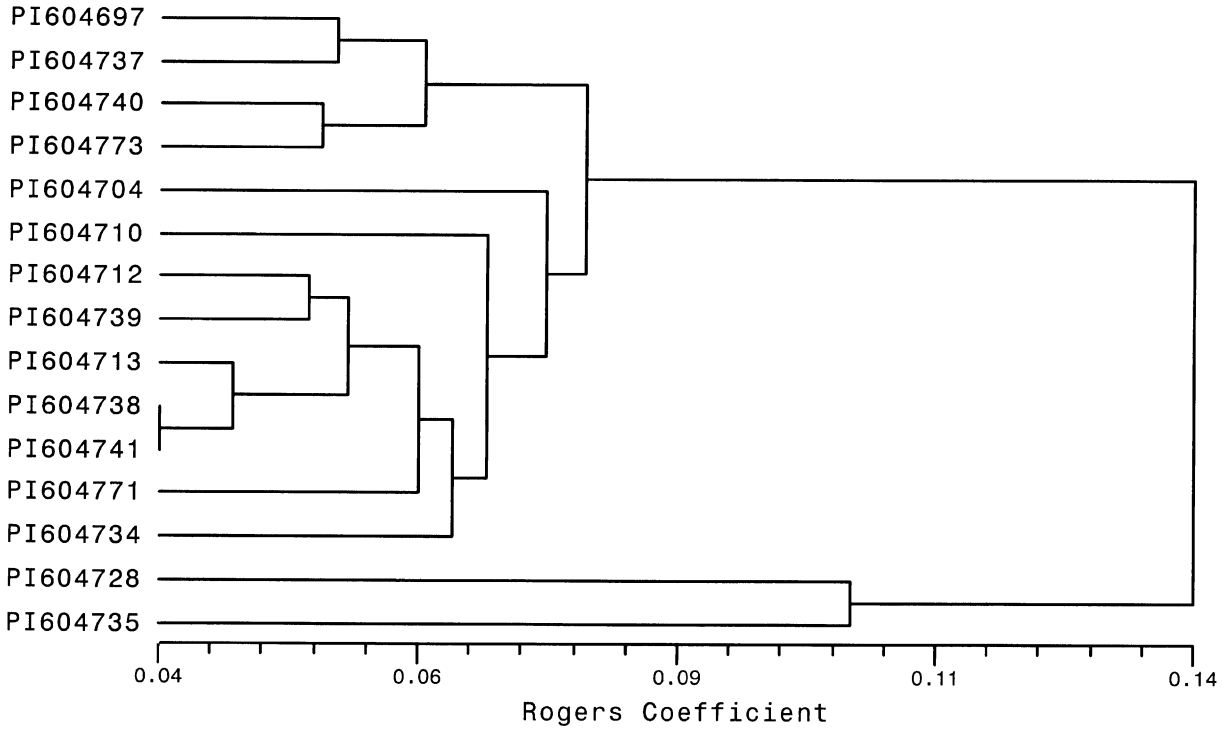

Fig. 1. Dendrogram of UPGMA clustering of 15 red clover populations from the Caucasus based on Rogers' genetic distance.

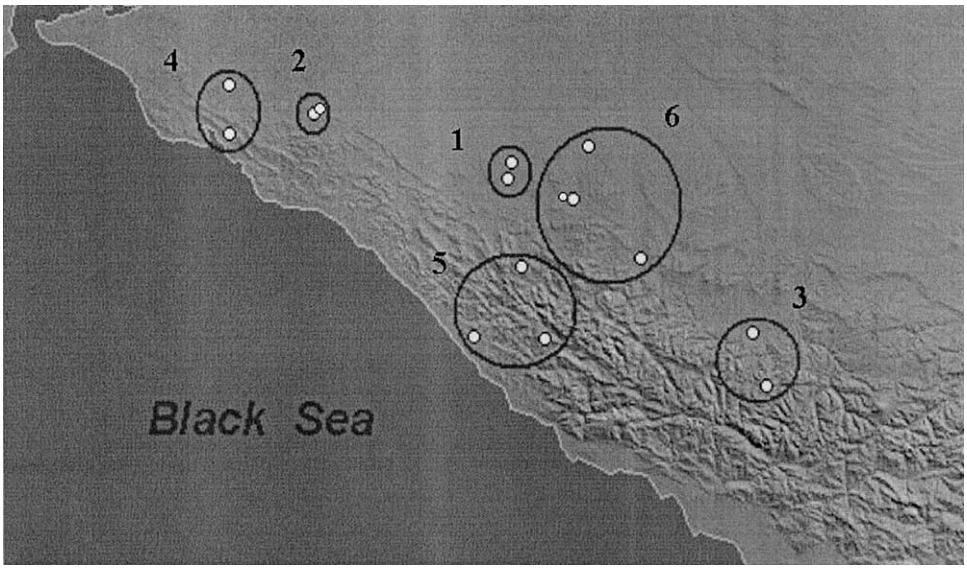

Fig. 2. Location of the collection sites in the Caucasus and grouping of the 15 red clover populations based on geographic proximity of the collection sites.

sites (average elevation was 1653 m) that had the most rainfall throughout the year (average annual accumulation of 1933 mm) and cold temperatures during the winter and growing season (average annual absolute minimum was −11.6°C). These two populations were found to have the greatest winter survival, the lowest percentage of first year flowering, the latest flowering date, and shortest duration of flowering of all populations when grown in Prosser, WA (Greene et al., 2004). The consensus tree confirmed the above grouping of the populations. Bootstrap numbers for the nodes indicated that PI 604738 and PI 604741 clustered together (bootstrap value of 61%); previously, they had been found to have a genetic identity of 1.00. The PI 604728 and PI 604735 also grouped together (bootstrap value of 55%) but separately from the rest of the other 13 populations. Those 13 populations were rather similar and were clustered in one group (bootstrap value of 67%). Furthermore, principal component analysis indicated that 59.2% of the variation was accounted by the first three dimensions. These results using isozymes suggested that PI 604728 and PI 604735 should be maintained separately, whereas the other 13 accessions could be consolidated into one accession by the National Plant Germplasm System.

Table 2. Mean gene diversity statistics for six groups of red clover populations. Parameters calculated for each group were percent polymorphic loci (*P*), mean number of alleles per polymorphic locus (*AP*), effective number of alleles per locus (A_e), observed heterozygosity (H_o), expected heterozygosity (H_e), Nei's coefficient of gene differentiation (G_{ST}) and gene flow estimates (Nm).

Group	Entries	*P*	AP	A_e	H_o	H_e	G_{ST}	Nm
1	PI 604737, PI 604738	90	2.67	1.88	0.392	0.375	0.020	12.07
2	PI 604734, PI 604739	80	3.00	1.72	0.320	0.317	0.031	7.74
3	PI 604728, PI 604741	80	2.88	1.83	0.367	0.371	0.220	0.89
4	PI 604710, PI 604740	80	2.63	1.77	0.347	0.344	0.029	8.53
5	PI 604704, PI 604735, PI 604773	70	2.86	1.76	0.334	0.331	0.249	0.75
6	PI 604697, PI 604712, PI 604713, PI 604771	90	2.56	1.82	0.359	0.342	0.042	5.77

Grouping the populations on the basis of geographic proximity yielded six groups (Fig. 2). Population genetic parameters for the six groups indicated that percent polymorphic loci (*P*) ranged between 70 and 80%, mean number of alleles per polymorphic locus (AP) between 2.56 and 3.00, effective number of alleles per locus (A_e) between 1.72 and 1.88, observed heterozygosity (H_o) between 0.320 and 0.392, expected heterozygosity (H_e) between 0.317 and 0.375, Nei's coefficient of gene differentiation (G_{ST}) between 0.020 and 0.220 and gene flow estimates (Nm) between 0.75 and 12.07 (Table 2). Group 1 had a very low level of gene differentiation as indicated by the G_{ST} value and a very high level of gene flow. This concurred with predictions based on GIS-derived maps that showed the two populations were collected from sites 7 km apart connected by valleys and with no obvious geographical barriers (Fig. 3a). Group 2 also had a low G_{ST} value and a high estimate of gene flow. Collection sites were only 1 km apart with no intervening land forms and had the same moisture and temperature regimes (Fig. 3b). Group 3 had a very high level of gene differentiation. The GIS-derived map showed that the collection sites were isolated by two ridges separated by an intervening valley with steep changes in elevation. The topography renders gene flow unlikely and explains the very low level of gene flow estimated (Fig. 3c). Collection sites for group 4 had no intervening barriers, had similar elevation, and sites were separated by 24 km; thus gene flow was possible. Results show a very low G_{ST} value and a high level of gene flow, thus agreeing with expectations (Fig. 3d). Sites for group 5 were separated by intervening mountains and hills. In addition, PIs 604735 and 604704 were separated by 23 km, PI 604704 was separated by 52 km from PI 604773, and PIs 604735 and 604773 were separated by 41 km. Results indicated a very high level of gene differentiation and a very low level of gene flow among the populations, again agreeing with expectations from the GIS-derived maps (Fig. 3e). In group 6, the collection site for PI 604697

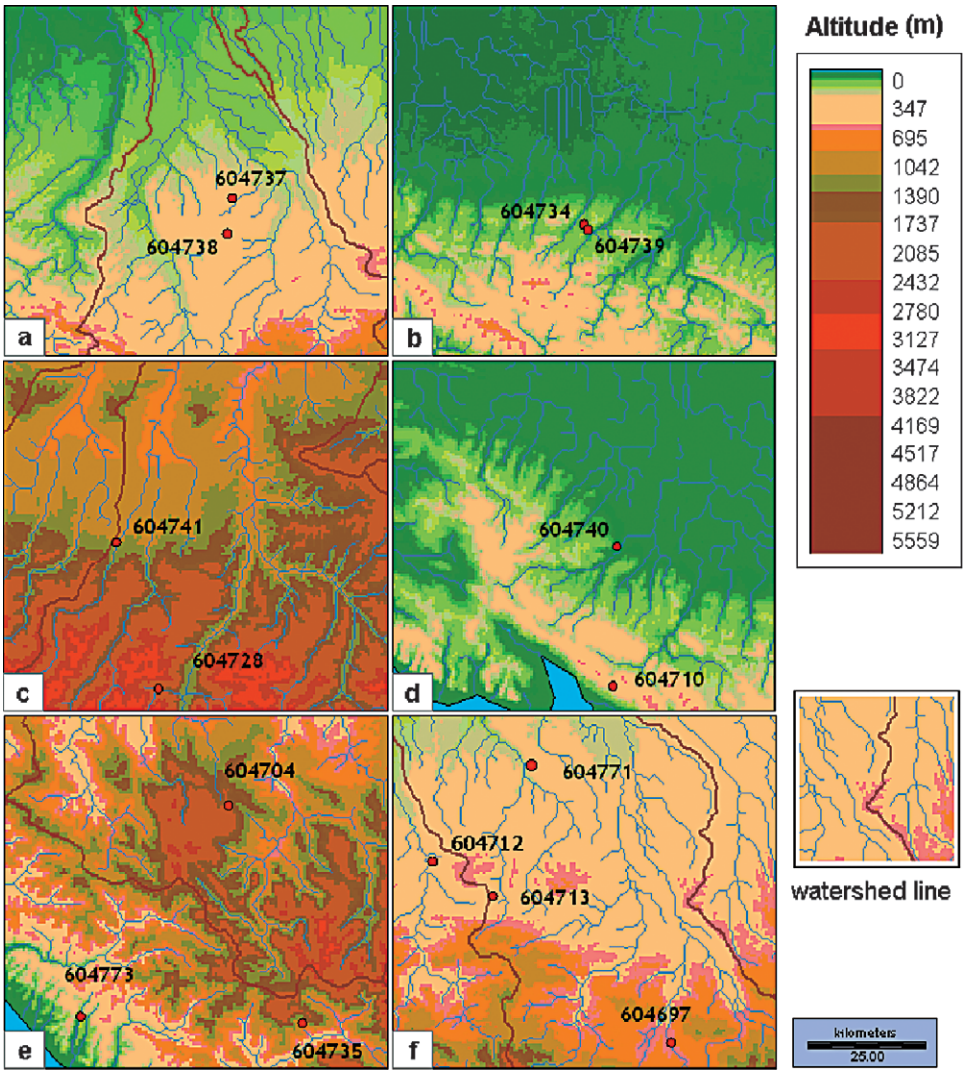

Fig. 3. Red clover collection sites plotted on a map illustrating elevation and watersheds: (a) group 1 sites were 7 km apart connected by valleys and with no obvious geographical barriers, (b) group 2 sites were only 1 km apart with no intervening land forms, (c) group 3 collection sites were isolated by two ridges separated by an intervening valley with steep changes in elevation, (d) sites for group 4 had no intervening barriers, had similar elevation and the sites were 24 km apart, (e) sites for group 5 were separated by intervening mountains and hills, furthermore, the sites were separated by distances that ranged between 23 and 52 km, (f) in group 6, some of the collection sites were isolated by distance and intervening hills whereas others were only separated by distance.

was isolated by distance (from 48–64 km) and intervening hills from the other populations. Populations 604712, 604713, and 604771 were in the same valley but separated by distance (15–32 km); consequently, some gene flow may be expected (Fig. 3f). Results indicated a low G_{ST} value and high gene flow. Reanalysis of the data after excluding PI 604697 from the group showed that the level of gene flow doubled, indicating that this population causes a lower gene flow estimate for the whole group, probably because of its physical isolation from the other populations in the group. Partitioning of genetic diversity indicated that most of the variability (90%) was within the populations, whereas the remainder (10%) was among groups. The value for variability within the populations was lower than that reported by Yu et al. (2001) suggesting that the collection was successful in finding genetic variability among accessions grouped based on geographic proximity. Variability among populations within geographic groupings was negligible.

In summary, diversity measurements of the Caucasus wild red clover populations confirmed previous studies with cultivated (Yu et al., 2001) and naturalized (Hagen and Hamrick, 1998) red clover that indicated that this species has a high level of variability. Some of the Caucasus populations such as PI 604728 and PI 604735 were unique at the at the isozyme level. The other 13 accessions could be consolidated into one accession that could be included in the red clover collection held by the National Plant Germplasm System. In every instance, gene flow expectations based on predictions using topographic maps developed from interpolated digital and climatic surfaces coincided with gene flow estimates obtained on the basis of isozymes. This supports the use of GIS tools and remotely sensed data to develop map

products that can support germplasm collection efforts and post-collection efforts to understand patterns of genetic diversity in collected germplasm. As expected, geographical barriers between populations limited gene flow between those populations. Conversely, gene flow was high between adjacent populations separated by many kilometers when no intervening land forms were present.

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